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Do the Inter-Nucleotide Domain loops act as an Entropic Sink in the Catalytic Activity of 3- Phosphoglycerate Dehydrogenase (3pGDH)?

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Proteins are amazing versatile molecules. Proteins spark the chemical reactions that are part of the basis for life. They transmit signals in the body, identify and kill foreign invaders, are part of the engines that make us move, and even record visual images. For a majority of tasks in a living organism, there is a protein specifically designed to perform each task.

Charles Tanford and Jacqueline Reynolds, authors of the Oxford press book, “Nature’s Robots: A History of Proteins” coined this beautiful big picture of proteins as the basis for life.¹ In any academic discipline, history serves the role of informing the status, progress and direction of a field of study. Since the inception of protein science in the late 19th century, much has been accomplished. The first amino acid, leucine, was discovered in 1819, and shortly thereafter, the nature of the peptide bond was revealed. This connecting bond revealed to scientists that proteins are made from a primary sequence of amino acids, that have a backbone, which forms hydrogen bonds around itself, forming alpha helices and beta pleated sheets. The specifics of the secondary structure were discovered through X-ray crystallography and other research techniques with the Nobel Prize going to Max Perutz in 1962 for solving the structure of hemoglobin.²

As the science advanced, the covalent structure of biologically active molecules, such as proteins and enzymes became readily available from enterprises such as genome sequencing project. As a result, protein sequences quickly became accessible. The downside was that the understandings of protein dynamics and structure couldn’t keep up

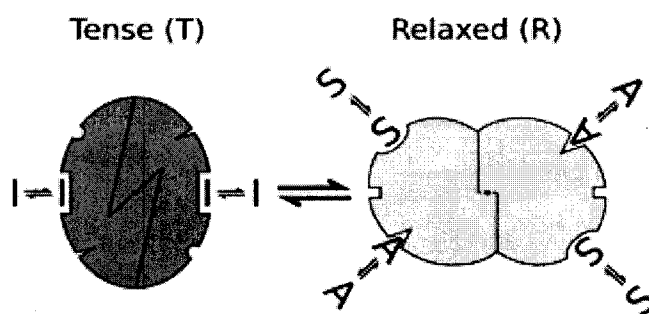
¹ Reynolds, Jacqueline. Tanford, Charles. “Nature’s Robots: A History of Proteins.” Oxford University Press Inc. New York. 2001. <<http://www.amazon.com/Natures-Robots-History-Proteins-Paperbacks/dp/019860694X>>

² Martz, Eric. Earliest Solutions for Macromolecular Crystal Structures. History of Visualization of Biological Macromolecules. <https://www.umass.edu/microbio/rasmol/1st_xtls.htm>

with the exponentially growing protein sequence database.³ These scientific constraints demanded new theories on the role that protein dynamics plays in the allosteric regulation of proteins.

In protein science the relationship between protein dynamics and catalytic activity are the subject of considerable contemporary interest. Although protein motions are frequently observed during ligand binding and release steps, the contribution of protein motions to the catalysis of bond making/breaking processes is more difficult to probe and verify. Currently, the field of protein science is trying to uncover the deeper role that dynamics plays in the catalytic activity and allosteric regulation of proteins and to better understand how to more effectively and efficiently target proteins in cancer therapeutics and other metabolic pathways.

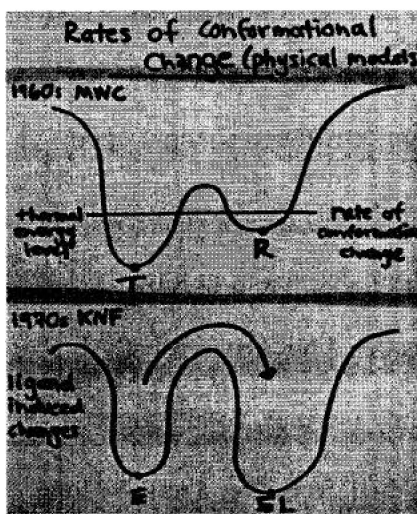
Historically there are two mechanistic models for protein allostery. The most basic model of protein catalysis is the lock and key model which states that there is a specific protein that binds a specific substrate with a certain affinity. This model was then studied more thoroughly by studying conformational changes. Monod Wyman and Changeux postulated, that enzyme subunits are connected in such a way that a conformational change in one subunit is conferred to all other subunits.⁴ So, in essence, all



³ Ponting, Chris. Russell, Robert. "The Natural History of Protein Domains." Department of Human Anatomy and Genetics, University of Oxford, MRC Functional Genetics Unit. Oxford, United Kingdom. Annual Review of Biophysics and Biomolecular Structure. Vol.31 45-71 June 2002.

⁴ MWC Model Wikipedia. http://en.wikipedia.org/wiki/MWC_model

subunits exist in the same conformation, but it holds true, that in absence of a ligand the protein favors one of the conformation states, T or R. T is for tense and R is for relaxed.⁵ This idea of two conformational states of hemoglobin is best portrayed through energy well diagrams. This model describes allosteric transitions of proteins made up of identical subunits. The conclusion of the MWC is that regulated proteins, enzymes and receptors, exist in different interconvertible states in the absence of a regulator.⁶



In the image to the left the energy well diagram depicts the conformational change from the T to R state. It is apparent that the R relaxed state has a lower energy well and is therefore possibly not as stable of a structure with less minimized energy. This model explained enzyme specificity, but it fails to explain the stabilization of the transition state that enzymes achieve.

In the 1970s, Koshland, Nemethy, and Flimer (KNF) suggested a modification to the R and T relaxed states. This model is a theory that describes cooperativity of protein subunits. They proposed the idea that since enzymes are rather flexible structures, the active site is continuously reshaped by interactions with the substrate, as the substrate interacts with the enzyme.⁷ The KNF theory was coined the “induced fit” theory. This induced-fit model was focused on the idea that when a ligand binds the ligand induces a

⁵ Duke TA, Le Novere N, Bray D (2001). Conformational spread in a ring of proteins: a stochastic approach to allostery. *J. Mol Biol.* 308: 541-553

⁶ Monod J., Wyman J., and Changeux J.-P. (1965). On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* 12: 88-118.

⁷ Changeux, J.P. Allosteric interactions interpreted in terms of quaternary structure. *Brookhaven Symposia in Biology* Vol. 17. Page 232-249.

certain conformational change in the structure of the protein. This uncovered the role of ligand binding in the conformational changes of a protein.⁸ So although the subunits change conformations independently the switch of one subunit makes the other subunits more likely to change, by reducing the energy needed for subsequent subunits to undergo the same conformational change.⁹ This is depicted with an energy-well in the lower half of the image above. There are two wells that show how the protein instead of only having two defined conformational states of R and T has a shift in conformational energy upon the binding of ligand.¹⁰ The enzyme-ligand complex has a distinctly different conformation with a separately minimized energy due to conformational changes upon ligand binding.

One of the limitations of these models is that they were derived from x-ray crystallographic analysis of proteins. The other limitation is that KNF explains both negative and positive cooperativity while MWC explains only the later. So although x-ray crystallographic images are an average of several individual shots over a relative long period of time, this method does not shed light on any role of protein dynamics in how a protein catalyses reactions or experiences allosteric regulation. Because of the static nature of x-ray imaging these historically accepted models are now evolving into new territory of trying to understand the contribution of protein motions to the catalysis of bond making and breaking and understand protein allostery more deeply.

The leap over these limitations has led to a new model coined, the dynamic type model. This dynamic model differs from the MWC and KNF models because it implies

⁸ Campbell, Mary and Farrell, Shawn. "Biochemistry" 7th Edition. Brooks/Cole Cengage Learning. Belmont, CA 2012. (169)

⁹ Koshland DE (February 1958). "Application of a Theory of Enzyme Specificity to Protein Synthesis". *Proc. Natl. Acad. Sci. U.S.A.* **44** (2): 98–104.

¹⁰ Monod J., Wyman J., and Changeux J. P. "On the nature of allosteric transitions: A Plausible Model." *Journal of Molecular Biology*. Vol 12. Pg 88-118. (1965).

that there are possibly an infinite number of dynamic conformations that a protein can transition through. Instead of having two distinct conformational energy wells, there would be an infinite number of possible conformations of a protein that all have equal probability of existing at the same minima. This greatly differs from a distinct R and T state that models two distinct conformations that are transitioned through upon ligand binding.

This new model has started to arise with a change of experimental techniques used to analyze proteins. The key to this change in experimental techniques came from a focus on techniques that gave dynamic information. Hydrogen/ Deuterium exchange, protein fluorescence, multi-quencher collisional quenching, molecular dynamics, x-ray crystallographic B-factor analysis and other experimental techniques have all produced dynamic information and open many doors for the dynamic type model.

To better understand this dynamic type model there are three definitions that are pertinent: protein dynamics, allostery and catalysis. Protein dynamics, as has already been touched on, is the idea that proteins are not strictly static objects, but rather populate ensembles of conformations. Transitions between these states occur on a variety of length scales and time scales. These transitions are most commonly conceptually synthesized with energy landscapes. Protein allostery is the binding of a ligand that affects the binding of another molecule to the protein structure or alters catalytic activity. Protein catalysis is the change in the rate of the chemical reaction that the protein carries out and a change in the energetics of the reaction.

This dynamic model opens the door to a whole new way of characterizing the importance of conformational changes in a protein's structure and how that informs

possible catalytic changes. These developments have superseded the historical models and by using new techniques to evade the constraints of x-ray crystallography. Protein dynamics has been accepted for its implications within protein catalysis, but only in the last four to five years has it started to shed light on possible allosteric considerations of protein structure-function relationships. The internal dynamics of enzymes have started to be linked with their mechanisms of catalysis. Internal dynamics, such as movement of individual amino acid residues, protein domains and groups of amino acids, can occur at time-scales from femtoseconds to seconds. These networks of internal dynamics within a protein can contribute to catalysis through domains motions. Although these movements are important in binding and releasing of the substrates and products, it has not yet been discovered precisely whether protein movements help to accelerate the chemical steps in enzymatic reactions. An understanding of protein dynamics can lead to a better understanding of allosteric effects and the development of new medicines that better target the abnormal cells.¹¹

While there is always an optimized conformation for a particular enzyme, some changes in protein conformation will not greatly affect the energetics of the system. A protein can change its conformation through a proposed continuum effect and such conformation flexibility is currently being investigated to understand what role it could play in the allosteric model of protein function and how this could affect catalysis of enzymatic reactions. This is where the idea of protein flexibility arises.¹²

¹¹ Dr. Ellis Bell Research Lab Introduction and Background. 3-pGDH Specific Aims, Experimental Techniques and Future Aims Grant Proposal.

¹² Kern, Dorothee. "Caught in the act: the dynamic dance of enzymes" <<http://www.brandeis.edu/now/2008/january/kernnature.html>> December 20th 2007.

Proteins are very dynamic molecules. Not only do they play a dynamic role in the foundations of life, they also exhibit a large range of dynamics in the interior of their structure. Proteins are generally thought to adopt unique structures determined by their amino acid sequences, however, proteins are not strictly static objects, but rather populate ensembles of conformations. Protein structures fluctuate on many different timescales: they can unfold (refold) and switch conformations. When transitioning between these states there is a large spectrum of time and length scales that have been linked to phenomena such as allosteric signaling and enzyme catalysis. There are three time scales to access protein dynamics. First local fluctuations in side chains on the pico and nano second time scale. The next group is domain movements such as alpha and beta helices on the milli-second time scale. The third time scale is overall conformational changes or global changes of the protein, on the milli-second to second time-scale. The study of protein dynamics is more specifically concerned with transitions between these states, but also involve the nature and equilibrium populations of the states themselves. With an interdisciplinary approach of kinetics and thermodynamics an energy landscape paradigm can be linked to the interpretation and explanation of protein dynamics.¹³

This new model is only recently arising as a possible explanation for protein allostery. However the dynamic model has for over ten years been of large debate in the circle of protein catalysis. The current debate in the literature is a battle between protein dynamics versus electrostatic pre-organization as a possible explanation for protein catalysis. The relationship between protein dynamics and function is a subject of considerable contemporary interest. The contribution of protein motions to the catalysis

¹³ Dr. Ellis Bell Research Lab Introduction and Background. 3-pGDH Specific Aims, Experimental Techniques and Future Aims Grant Proposal.

of bond making/breaking processes is a difficult mechanism to probe and verify. There is a new emerging theory that electron tunneling could be a possible explanation for how to explain the role of protein dynamics in catalytic processes.

This unique explanation through quantum mechanical hydrogen tunneling and its association with enzymatic C-H bond cleavage provides a unique window into the necessity of protein dynamics for achieving optimal catalysis.¹⁴ Due to persistent deviation of empirical observations for enzymatic C-H activation from semi-classical predictions led to a heightened popularity in incorporating the QM nuclear tunneling into theoretical models. Tunneling is a phenomenon in which a particle crosses a barrier between reactants and products due to its wave-like nature. This phenomenon occurs when the probability of finding an electron in the products and reactants is equal. There are necessary motions of heavy atoms within the protein structure whose movement is essential for reorganization to bring the donor and acceptor from the reactant's ground state to the tunneling-ready states.¹⁵ This theory is accepted for its model that shows how protein dynamics and flexibility are important for the formation of a lid-closure form active site in which the hydrophobic regions of the active site are enclosed are separate from the aqueous environment.

Dihydrofolate synthase has been an enzyme at the center of the debate between protein dynamics and electrostatic pre-organization. The field is split between the electrostatic preorganization theory versus the dynamic model to explain protein catalysis. Andrew Adamczyk, of the University of Southern California, published an

¹⁴ Klinman, Judith. Kohen, Amnon. "Hydrogen Tunneling Links Protein Dynamics to Enzyme Catalysis." Annual Review of Biochemistry 2013.

¹⁵ Klinman, Judith. Kohen, Amnon. "Hydrogen Tunneling Links Protein Dynamics to Enzyme Catalysis." Annual Review of Biochemistry 2013.

article in 2011, *catalysis by dihydrofolate reductase and other enzymes arises from electrostatic preorganization not conformational motions*. This article reports that several mutations in the active site of the dihydrofolate synthase enzyme were designed and the change in catalysis upon these mutations was measured. With different mutations came changes in the distance and dynamical orientation between the donor and acceptors of the catalytic reaction. Conclusions were made by calculating change in corresponding activation barriers without the need to invoke dynamical effects. This data was compounded with catalytic landscape of the enzyme, which demonstrated that motions in the conformation space do not help drive catalysis. This paper argues for the imperative role of electrostatic preorganization in catalysis and that protein dynamics is never the reason for catalysis but rather simply a reflection of the shape of the reaction potential surface.¹⁶ This study showed that even with the mutations within the active site the catalytic power of the enzyme was maintained because electrostatic preorganization capabilities were retained. The loophole in the dynamic model is argued to be a misunderstanding of how to study electrostatic preorganization and therefore a major lack of consideration for certain phenomena in protein catalysis. Previously, people believed that electrostatic preorganization could be determined from examining experimentally based X-ray or NMR structures; however these techniques have shortcomings and don't effectively convert structural information to the corresponding reorganization energy, which the researchers believe can only be quantified by microscopic approaches.¹⁷

¹⁶ Adamczyk, Andrew. "Catalysis by dihydrofolate reductase and other enzymes arises from electrostatic preorganization, not conformational motions." PNAS July, 12th 2011.

¹⁷ Warshel, Arieh. "Electrostatic Origin of the Catalytic Power of Enzymes of the Role of Preorganized Active Sites." The Journal of Biological Chemistry 273. October 16, 1998.

Another study released in 2013 by Louis Luk from the Cardiff Catalysis Institute argues that *unraveling the role of protein dynamics in dihydrofolate reductase catalysis* is a very complex and taxing endeavor. In this paper, studies of quantum mechanics/molecular mechanics simulations and theoretical analyses seek to identify the origins of the observed differences in reactivity. This data showed that there is generally a slightly slower reaction in heavy enzyme, which reflects differences in environmental coupling to the hydride transfer step. Furthermore, this paper argued that since the barrier and contribution of quantum tunneling were not affected, there was no significant role for “promoting motions” in driving tunneling or modulating the barrier. In conclusion, the heavy atom substitution experimentations of dihydrofolate reductase catalysis presented a minimal support for the role of protein dynamics role in protein catalysis with a measurable but small effect on the chemical reaction rate.

Two different models K-type and V-type can characterize effects on protein functioning. K-type effects are changes in the binding affinity for the substrate to the active site or a ligand to a binding site. The K-type corresponds to the binding energy because K_m is the relative strength of the binding affinity between ligand and enzyme binding site. If K_m is increased than that corresponds to a relative decrease in binding affinity and if the K_m is decreased inversely is corresponds to increased binding affinity following general Michaelis-Menten kinetics. The K_m is affected with the conformational change and dynamics of the protein structure-function relationship. K-type enzymes have a fairly constant V_{max} if at a fixed cellular concentration of substrate. As their affinity is made poorer their rate must be slower and as their affinity is improved, their rate will be faster. It is important to think back to the basics of protein conformation to understand

how the alterations in V_{\max} and K_m arise. V-type effects are associated with the activation energy because it corresponds to the velocity of the reaction and the V_{\max} of the reaction, which is the max occupied active sites in the catalysis of the reaction. Altering the catalytic capabilities of a protein at its active site and altering the V_{\max} of the reaction, characterize V-type effects. These effects are more associated with fast-acting proteins that carry out their enzymatic activity at a fast rate in which the V-type effect would trump the K-type effect.¹⁸

The turnover rate, the speed of catalysis, is important because it relates back to the protein dynamics time-scales and an understanding of protein dynamics on the time-scale, which correspond to the enzymatic activity of a protein. A slower turnover rate and completion of the catalytic cycle corresponds more to the millisecond second time scale, where as a medium turnover number is around the micro to milli time scale and fast enzymes are on the pico and nano time scale. This is significant because a fast enzyme would have more local flexibility and conformational changes due to rearrangements of amino acid side chains. For a medium to slow enzyme the micro to second time scale will be significant and will also inform whether the protein displays V-type or K-type effects.

19

The next step in the newly proposed dynamic type model and the novelty of this research is the idea of an entropic sink. First entropy is the common term from general chemistry that was introduced in these two following equations:

$$\Delta G = \Delta H - T\Delta S$$

$$\Delta G = -RT \ln K_{eq}$$

¹⁸ Bell, Jessica. Grant, Gregory. Banaszak, Leonard. "Multi-conformational States in Phosphoglycerate Dehydrogenase" Journal of Biochemistry American Chemical Society. January 23, 2004.

¹⁹ Thompson, James. Bell, Jessica, Bratt, Judy. Grant, Gregory. Banaszak, Leonard. "Vmax Regulation through Domain and Subunit Changes. The Active Form of Phosphoglycerate Dehydrogenase." Biochemistry. February 22, 2005.

These equations are important because they create a link between protein catalysis, allostery and dynamics. The idea is that if there is a free energy change within a protein system, that change is either the result of an enthalpic or entropic variable.²⁰ This is directly seen in the equation to the left. In deciphering the enthalpic versus entropic considerations in a free energy change there is great consideration in K and V type changes. If a protein exhibits K-type changes there is less focus on the active site of the protein and therefore less of an enthalpic argument. This is because the enthalpic changes within a protein are usually associated with energy needed for catalysis and carrying out the chemical reaction of interest for the specific protein. The entropic effects have been less explored with little thought going to the idea that changes in flexibility and dynamics in several parts of a protein could be a possible link to a change in free energy at a different site within a protein. The key to the entropy sink concept is that there is a highly flexible region of the protein that produces energy changes required for increased or decreased binding or catalysis. Therefore the idea of an entropic sink can be applied to both allostery and catalysis concepts. This is seen through the thermodynamic equations and controlling the entropy term in the free energy equation.

This idea of entropy for a protein is explained through conformational flexibility and the protein's dynamics.²¹ For example, a protein that has a possible entropic sink is a protein that contains a highly concentrated flexible region within its structure that generates high amounts of entropy for the system and therefore greatly dictates the free

²⁰ Villa, J. Strajbl, M. "How important are entropic contributions to enzyme catalysis?" PNAS Aug. 21 2000 < <http://www.pnas.org/content/97/22/11899.full.pdf>>

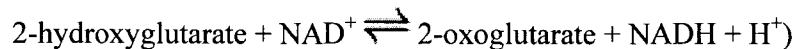
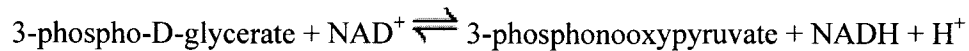
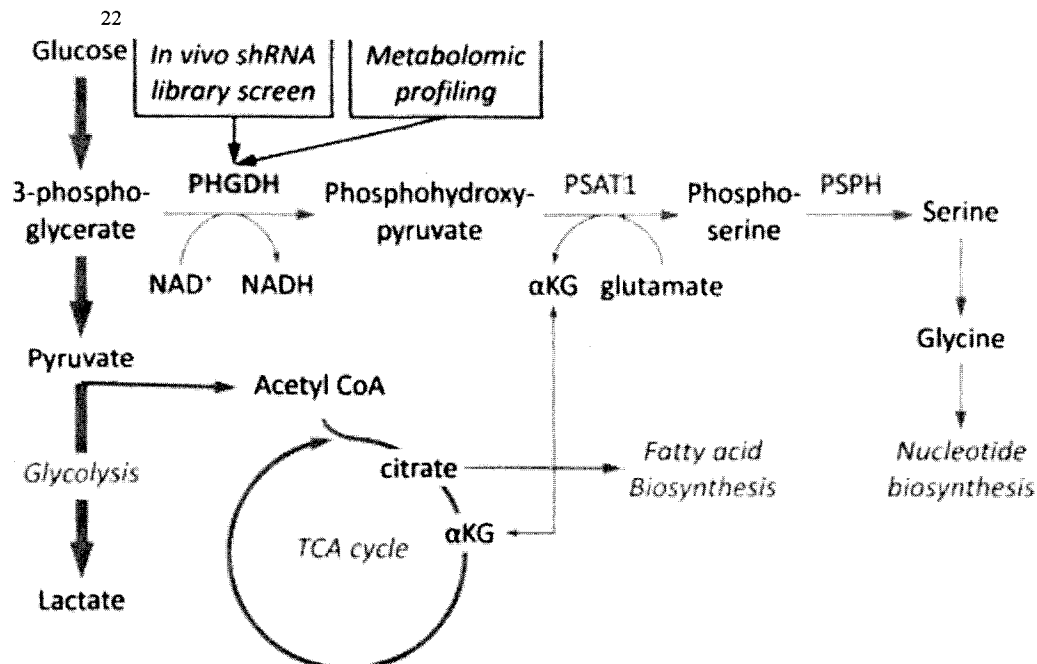
²¹ Popovych, Nataliya. Sun, Shangjin. Ebright, Richard. "Dynamically Driven Protein Allostery." Nature Structural & Molecular Biology. Aug. 13th 2006. < <http://www.nature.com/nsmb/journal/v13/n9/full/nsmb1132.html>>

energy of the system. This change in free energy is then related to the equation on the left above that shows that a change in free energy can have many effects on the protein system. Most specifically, changing the binding affinities within the protein changes how ligands bind to several different domains within the interior of the protein. K_{eq} is generally known as the ratio of products/reactants and in the binding events of $L + E \rightarrow EL$ complex a greater K_{eq} means a greater concentration of EL complex and more-tight binding of the ligand. So to relate this idea back to the historical models of MWC and KNF, neither of these models propose an explanation for energy differences that arise from molecular level dynamics and changes and no one has ever proposed the idea of an entropic sink.

Therefore the model system that was chosen in this study to bring together this idea of protein catalysis, allosteric regulation and entropy was 3-pGDH. 3-pGDH was a good choice of a model system because it has been well characterized, both biochemically and structurally. 3-pGDH is a Dehydrogenase Enzyme. Dehydrogenases belong to the group of oxido-reductases that oxidize a substrate by a reduction reaction that transfers one hydride to an electron acceptor NAD^+ . 3-pGDH more specifically belongs to a family of oxido-reductases that act specifically on the $-CH-OH$ group of donors with NAD^+ or $NADP^+$ as an acceptor.

3-pGDH is a homo-tetrameric protein that plays an important catalytic role at a branch point of several pathways including glycolysis, TCA cycle, fatty acid biosynthesis and nucleotide biosynthesis. This pathway is depicted in the image below and is allosterically inhibited by the pathway's end product, serine:

:



There are two reactions listed here because the 3-phospho-D-glycerate is the in vivo substrate and 2-hydroxyglutarate is the in vitro substrate that is used in lab for experimentation. In vivo 3-pGDH catalyzes the inter-conversion of 3-phosphoglycerate and phosphor-hydroxypyruvate using NADH. This reaction is regulated by serine because following the production of serine there are many important downstream products such as tryptophan. When there are high levels of serine, serine acts as a feedback inhibitor of 3-pGDH serving its role in a negative feedback mechanism. This inhibition by serine is a very important regulation mechanism to control the production of some of the most important biosynthetic reactions of anabolism and catabolism in this

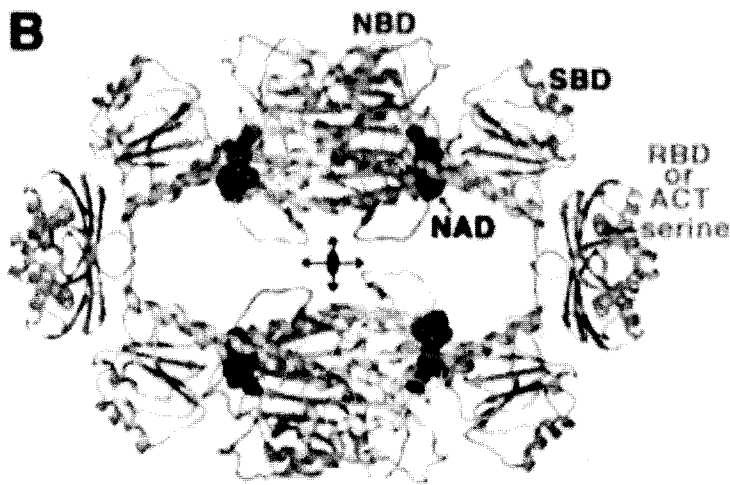
²² Luo "Serine Biosynthetic Pathway" Breast Cancer Research 2011 13:317. < <http://breast-cancer-research.com/content/13/6/317/figure/F1?highres=y> >

metabolic pathway imaged above. The regulation by serine is understandable, because the enzymatic activity of 3-pGDH is a precursor reaction to TCA cycle, fatty acid biosynthesis, nucleotide biosynthesis, and glycolysis. The serine regulation of 3-pGDH serves as a metabolic gatekeeper both for macromolecular biosynthesis and serine-dependent DNA synthesis. The inhibition by serine was a second ideal reason to use 3-pGDH as a model, because 3-pGDH is an allosteric-regulated system.

To make the link between protein dynamics, catalysis and allostery for 3-pGDH it is important to define the catalysis and allosteric regulation of 3-pGDH. Enzyme catalysis: the increase in the rate of 3-pGDH's chemical reaction converting 3-phosphoglycerate to phosphohydroxypyruvate. Conventionally an enzyme lowers the activation energy of a reaction, which increases the amount of reactant molecules that achieve a sufficient level of energy to reach the activation energy. Allosteric regulation for 3-pGDH deals with the binding of a serine molecule affecting the binding of cofactor, NADH at a distinctly different site altering the K_m cofactor binding at the nucleotide-binding domain. These definitions and the fact that 3-pGDH is a slow enzyme characterize these changes as K-type effects.

An extension of this binding discussion led to the idea that in multiple subunit domains binding, enzymes display cooperative binding. Cooperative binding is the idea that the binding of a ligand in one subunit can affect the binding affinity in another. This is apparent when looking at the structure of 3-pGDH. This form of 3-pGDH shows 4 bound molecules of the substrate NAD and the regulatory molecule serine. (NAD in

black and serine in pink-in illustration below).

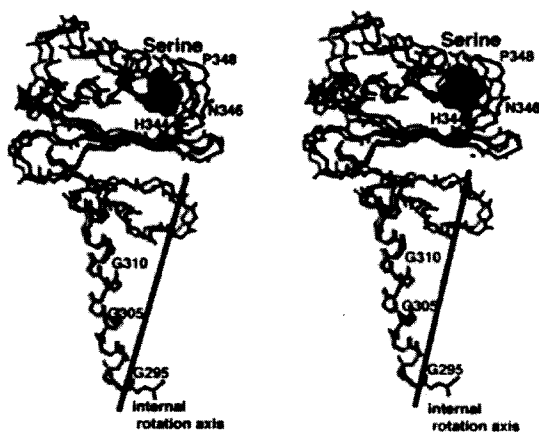


Upon the binding of the allosteric molecule serine, there is a proposed hypothesis that the binding of this ligand leads to dynamic conformational changes within the protein. These ligand induced conformational changes are thought of as action-at-a-distance. This communication is translated into the cooperativity that 3-pGDH has displayed in binding studies. In the homo-tetrameric structure the first two molecules that bind display positive cooperativity and the second two molecules display negative cooperativity. This means that when the first ligand binds it positively increases the binding affinity for the second ligand, yet consequently, when the third molecule binds, it binds less tightly and the fourth less tightly.

This suggests a possible pairing of energetics for the four subunits in their paired binding patterns. This idea of cooperativity is similar to a sigmoidal dependence on the concentration of a substrate with small concentrations of an effector molecule causing the enzyme to bind its ligand with different affinities. A redistribution of binding energy explains the transition between the higher affinity and lower affinity states. The most common example of this is the sigmoidal binding curve for the common component of

blood, hemoglobin. The current suggested weaknesses in this theory are the idea that maybe protein dynamics play a larger role in the allosteric activity of enzymes than previously thought and that the model for protein dynamics is not simply a ligand induced change, but a more continuum effect of conformational flexibility. The idea of the changing conformation of 3-pGDH upon binding of serine is displayed in the images below with superimposed x-ray crystallographic black images of 3-pGDH being without serine and the grey image corresponding to the serine-bound form. It is apparent from the image below that the P348 region displays a significant conformational change upon the

binding of serine.



This project has been solely focused on 3-Phosphoglycerate dehydrogenase and understanding the important role of dynamics in regulation of the activity of 3pGDH, as well as the established roles

for subunit interactions. Protein dynamics plays an important role in information transmission, from ligand binding triggering conformational changes followed by the transmission of this impulse through the protein and across subunit interfaces to elicit an effect²³. This work investigates the effect of regulatory ligands on the conformation of the interface between adjacent domains. Studying the interface between adjacent domains can lead to a better understanding of in cross-subunit communication and cooperativity. The conformational change provides a mechanism by which ligand binding to one

²³ Dr. Ellis Bell Research Lab Introduction and Background. 3-pGDH Specific Aims, Experimental Techniques and Future Aims Grant Proposal Manuscript.

subunit can increase the interactions between adjacent subunits and thus their communication.²⁴

3pGDH exhibits cooperative binding of NADH (positive cooperativity for the first two molecules and negative cooperativity for the second two). This was studied more extensively once the crystal structure was solved and the x-ray crystal structures of the serine-free form of the enzymes were compared with the ligand (serine) bound form to show the differences in structure in the presence of the binding ligand. No crystal structure of 3pGDH apo-enzyme has been forthcoming because of the high affinity for cofactor. This precludes comparisons of apo-enzyme and cofactor bound enzyme of the type that have been possible for plus and minus serine binding (in the presence of cofactor). However the limited x-ray crystallographic studies gave more information for the changes that occur in the binding of the ligands and uncovered more information of how the specific binding and catalytic mechanism of 3-pGDH occurs.²⁵

This information ties back to the bigger question of deciphering the physical basis of long-distance communication through proteins. By studying the serine bound form and serine free form of 3pGDH, a hypotheses can be proposed for the mode of action for communicating serine inhibition through the 35-40 Å (angstrom) distance from the allosteric site to the distant active site. This communication until recently has been long attributed to large-scale hinge motions across domains coupled with residue-level motions within each domain.

Our research is now focused on coupling residue-level displacements with calculated large-scale, rigid-body motions that could account for a complete network

²⁴ Falk, Bradley and Bell, Ellis. "Probing Subunit Interactions in 3-Phosphoglycerate Dehydrogenase" Department of Chemistry University of Richmond Manuscript.

²⁵ Bell, Jessica. Pease, Paul. Bell, Ellis. Grant, Gregory. Banaszak, Leonard. "De-regulation of D-3-phosphoglycerate dehydrogenase by domain removal" Eukaryotic Journal of Biochemistry. 269. 4176-4184.

explanation for structural changes that occur upon inhibition. The role of changes in protein dynamics was indicated in further exploration of the ACT-domain family of proteins that found that there may be a reduction of flexibility within the ACT domain alone upon ligand binding.

The ACT domain of proteins is all ferredoxin like topology and have 8 stranded antiparallel sheets with two molecules of allosteric inhibitor serine bound in the interface. The regulatory binding domain of 3pGDH is a member of the ACT-domain family. A novel approach using B factor analysis and complementary experimental approaches was utilized to study this domain within the structure of 3pGDH and its role in ligand binding. This novel approach was used to determine the impact of serine binding upon protein stability and dynamics and the ACT domain's role in allosteric inhibition of a V-type enzyme.

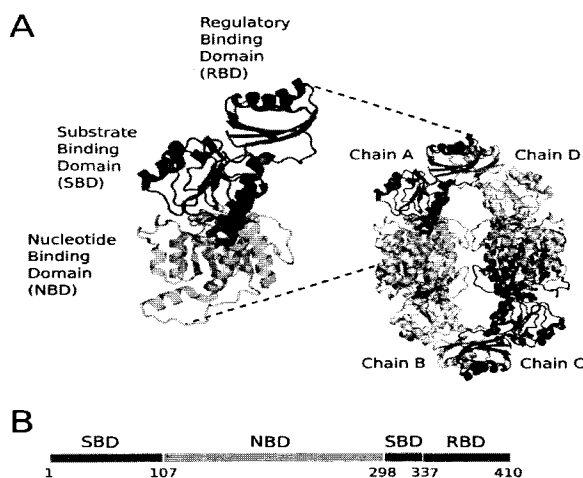
To greater understand the role of global and domain protein dynamics several experimental techniques have been utilized. The experimentation in this project was focused on the idea that the active site contains residues acting as a trigger for ligand induced conformational effects. The big picture is to understand the critical role played by ligand induced changes in the subunit interfaces. There are discrete pathways of coordinated motions that relay these effects through a subunit and the molecular switches at subunit interfaces transmit flexibility changes to an adjacent subunit. Specific techniques were utilized to identify the trigger residues in the active site of each of the enzymes, which initiate the flow of dynamic information to and across the subunit interfaces. These techniques include: site directed mutagenesis and enzyme kinetics in conjunction with truncated QM-MM calculations together with direct measures of

functional subunit interactions. To determine whether there is an explicit linkage between folding/unfolding cooperativity and allosteric behavior the correlation between ligand binding events and cooperativity. Site directed mutagenesis was utilized to alter the cooperativity, which had consistent effects on cooperative unfolding. This was measured by the steepness of the unfolding transition in either thermal melts followed by CD or chemical denaturation followed by fluorescence and dynamic light scattering. Since there is already a link established between overall stability of the protein and optimal activity and allosteric behavior, it will be informative to see whether this link extends to unfolding cooperativity, which will provide additional insight to the multiple roles amino acid side chains may play in protein structure and function relationships.

These approaches are seeking to shed light on the role of the active site and second sphere residues in triggering allosteric phenomena as well as validating the computational approaches used. For 3-pGDH, the substrate site contains R240, R60, R62, K39 and K141, based upon the available crystal structures. While extensive studies have been done on the active site of the substrate binding and cofactor binding domains, the focus of this paper is to characterize the loop region of phosphoglycerate dehydrogenase and how this loop region changes with ligand induced changes in conformational flexibility. Previous site directed mutagenesis studies have uncovered specific intriguing regions of the 3-pGDH structure.²⁶ The substrate-binding domain (residues 13-63 increased: residues 7-13 decreased) in the nucleotide binding domain (residues 103-127 and 160-230 both increased) and the regulatory binding domain (residues 389-410 increased. 335-337 increased, 348-349 decreased, 374-384 decreased and individual

²⁶ Falk, Bradley and Bell, Ellis. "Probing Subunit Interactions in 3-Phosphoglycerate Dehydrogenase" Department of Chemistry University of Richmond Manuscript.

residues 340 ad 356). The 160-230 region is particularly intriguing since it encompasses the X interface connecting subunits A & C and B & D. This X interface is the center of my research.²⁷



The goal in studying the X interface of the protein is to test the hypothesis of this project that this loop region serves as an entropic sink in the catalytic activity of 3-pGDH. The goal of the bigger research question is the linkage between allosteric interactions, catalysis and entropy. To shed light on the role that

protein dynamics plays in protein catalysis and allostery, we will investigate the question “Do the Inter-Nucleotide Domain loops act as an Entropic Sink in the Catalytic Activity of 3-Phosphoglycerate Dehydrogenase (3pGDH)?”

The first experimental technique utilized to study 3-pGDH was site-directed mutagenesis. Mutants were designed based on genetic clustal analysis. Several forms of 3-pGDH were compared from prokaryotes and eukaryotes. In comparing the prokaryotes and eukaryotes the consistency of conservation can be confirmed. The residues that have been conserved over evolution must be conserved because of their significant importance in the functioning of 3-pGDH. This clustal is depicted in the image below.

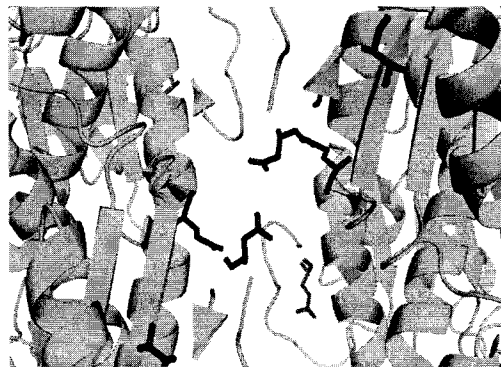
²⁷ Dr. Ellis Bell Research Lab Introduction and Background. 3-pGDH Specific Aims, Experimental Techniques and Future Aims Grant Proposal Manuscript.


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gi|2674062|gb|AAB88664.1|      KTLGILGLGRIGREVATRMQSFGMKTIGYDPIISPEVSASFVQQLPLEE 195
gi|52782263|dbj|BAD51978.1|    KTLGILGLGRIGREVATRMQSFGMKTIGYDPIISPEVSASFVQQLPLEE 195
gi|7688285|emb|CAB89828.1|      KTLGILGLGRIGREVAARMQAFGMKTIGYDPIISPEVAASFVQQLPLEE 195
gi|308174098|ref|YP_003920803. KTLGIIIGLGRIGSEIAQKARAFGMTVHVFDPFLEERAGKIGVNSRTFEE 188
gi|194368789|pdb|3DDN|A         KTVGVVGLGRIGQLVAQRIAAFGAYVVAYDPYVSPARAAQLGIELLSLDD 191
gi|117958135|gb|ABK59388.1|    KTLGIIGYCHIGSGLSVLAELHVLVYDVTIMALGTAR--QVSTLDE 246
gi|320583160|gb|EEW97376.1|    KTLGIIVGYCHIGSGLSVLAELHVLVYDVTIMALGTAK--QVSSLNE 247
gi|330840994|ref|XP_003292491. KSLGIIGYCHIGSGLSVLAELHVLVYDVTIMALGTAK--QVSSLNE 199
gi|218061147|emb|CAT81422.1|    KTLGIIGYCHIGSGLSVLAELHVLVYDVTIMALGTAK--QVSSLNE 199
gi|66360693|pdb|1YBA|D         KTLGIIGYCHIGSGLSVLAELHVLVYDVTIMALGTAK--QVSSLNE 199

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This clustal is a comparison of prokaryotic forms specifically because the prokaryotic form of 3-pGDH is self-regulating. So in finding which amino acids are conserved within the prokaryotic form, the specific amino acids involved in the self-regulating capabilities of 3-pGDH can be identified. Again these self-regulating residues are important because they are the possible location of the entropic sink in the interior loop region of 3-pGDH. Based on



these genetic clustals one mutant that was generated was E171Q (glutamine to glutamate). This mutation is significant because it is a mutation of a negative charge to a neutral charge so in the interior region of the protein where the loops are interacting there could be a hypothesized decrease in entropy because there is less repulsion from the deletion of the negative charge repulsion of the glutamines. This was confirmed in kinetic

activity assays conducted on the E171 mutant.

Kinetics Comparison of Native and E171Q		
	Native	E171Q
Activity at Saturating Substrate Concentrations (No Serine)	0.050 ± 0.001*	0.003 ± 0.0001*
Activity at Saturating Substrate Concentrations (+ Saturating Serine)	0.003 ± 0.001*	0.004 ± 0.001*
<p>For the kinetics the same concentration of Native and E171Q was used so the data was directly compared. The serine was saturating since similar data was obtained at three different concentrations of serine. *ΔA₃₄₀/Min</p>		
<p>Conclusions E171Q showed a twenty-fold reduction in activity compared to the native. E171Q showed no inhibition by serine where as the native showed 95% inhibition inhibition.</p>		

In this activity assay it is shown that with the addition of serine, the native form of 3-pGDH displays a decrease in activity from 0.050 to 0.003. This serine inhibition is disabled with the E171Q mutant, as there is not significant activity difference in the presence of serine. This loss of serine inhibition as well as the relative decrease in overall activity of 3-pGDH is telling of the possible loss of entropy from the mutation. Since the negative charge repulsion was eliminated, the system could possibly create less entropy in the central loop region interface. This connects back to the thermodynamic argument that less free energy in the system could mean that there is less tight binding of the co-factor NADH to the nucleotide binding domain.

The next experimental technique that was utilized was multi-quencher collisional quenching. Fluorescence quenching is a process, which decreases the intensity of the fluorescence emission. The accessibility of groups on a protein molecule can be measured by use of quencher to perturb fluorophores. The accessible fluorophores experience a decrease in fluorescence upon collision with collisional quenchers. The three quenchers of interest were acrylamide (neutral), iodide (negative), and cesium

(positive). Acrylamide is effective in giving information about local flexibility and accessibility of fluorophores within the protein structure. Cesium and iodide generate a bit more complicated of data giving a mix of information about local flexibility but more importantly the local charge environment around the fluorophore.²⁸ The fluorophore of interest is the single tryptophan in the structure of 3-pGDH. The following data is from a full-set of quenching experiments:

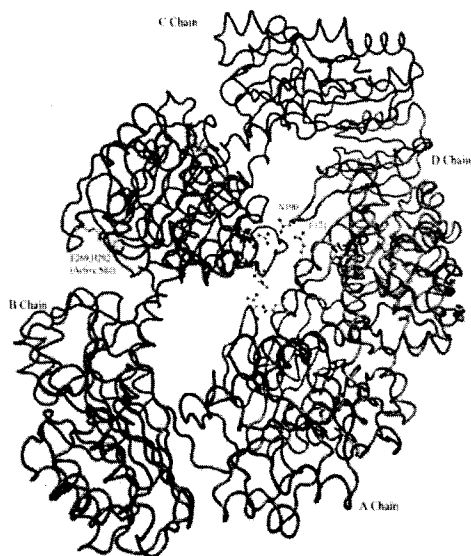
Wavelength	Stern Volmer Slopes	Standard Error	Stern Volmer Slopes	Standard Error	Stern Volmer Slopes	Standard Error
	pGDH Acrylamide		pGDH Iodide		pGDH Cesium	
330 nm	120.5181	4.2051	43.1485	2.1986	37.5305	1.042
335 nm	118.9049	5.2431	38.7937	2.3869	37.9858	0.7338
340 nm	113.4146	4.0898	38.4883	2.9222	36.509	1.1855
345 nm	120.3243	4.6663	35.4125	2.6739	37.2132	0.977
350 nm	117.6418	3.7183	33.5996	2.648	38.6798	1.3599
	pGDH aKG Acrylamide		pGDH aKG Iodide		pGDH aKG Cesium	
330 nm	122.8359	2.1828	43.8667	0.7128	30.8271	0.7394
335 nm	124.8772	4.1231	42.8007	1.1813	31.2002	0.9393
340 nm	124.0154	2.125	42.3648	1.0054	30.3249	1.2742
345 nm	114.7493	3.6455	43.0793	1.1885	29.129	1.0937
350 nm	111.0584	3.2912	42.3815	0.6384	29.4148	0.9672
	pGDH Serine Acrylamide		pGDH Serine Iodide		pGDH Serine Cesium	
330 nm	100.9137	4.4971	37.0271	1.0821	38.3121	1.9387
335 nm	90.4263	3.322	37.074	0.8883	35.9523	1.9664
340 nm	85.8272	4.6474	37.0991	0.9379	37.2458	1.5295
345 nm	89.8729	3.593	34.5322	1.1874	38.054	1.8441
350 nm	94.3224	4.2092	34.3093	0.9163	38.1579	1.774

There is a lot of data in this chart but there a few essential conclusions.. In comparing the pGDH acrylamide samples from the *acrylamide and p-gDH* and *pgDH Serine Acrylamide* data sets it is apparent that in the presence of serine the quenching data is decreased which is correlated with a decrease in flexibility in the region where the fluorophore is present. This is significant because it shows that upon the binding of serine the overall

²⁸ Mcoz, G. "Quenching of Fluorescence to Measure Accessibility of Tryptophan Residues" A PPS'99 Project. <
http://dwb.unl.edu/Teacher/NSF/C08/C08Links/pps99.cryst.bbk.ac.uk/projects/gmocz/qch.htm>

flexibility of the protein is decreased and rigidified in this binding event. This same pattern holds true for the iodide data. However in the cesium data there is a different trend that in the presence of serine the quenching data is increased. This goes back to the complexity of iodide and cesium data giving information about the local flexibility as well as the local charge environment. Furthermore, this data is not necessarily indicating that the local flexibility around the tryptophan decreases but possibly that there are changes in local charge environment.

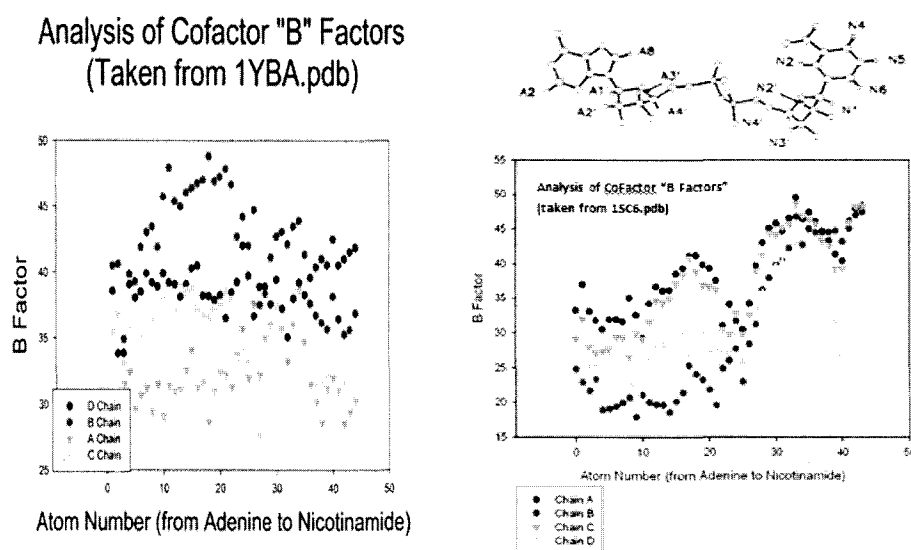
This data was then followed with a new direction in the research, exploring the possible conclusions from b-factor x-ray crystallographic information. A b-factor is an indication of the mobility of an atom in a macro-structure. Protein structures are not fixed static structures and experience dynamic transitions between conformations and



local and global flexibilities. So an atom with a high b-factor is an atom that experiences high mobility within the structure of 3-pGDH. This study was focused on the nucleotide-binding domain of 3-pGDH and a measure of the relative motility for the atoms from this domain that associate with the co-factor NADH.

Furthermore, there was a direct quest to compare the differences in motility between the A and C and B and D subunits to support the previous studies that showed a positive and negative cooperativity exhibited by the binding of NADH to the nucleotide binding domain. In the following *Analysis of*

Cofactor "B" Factors graphs it is apparent that there are four nucleotide-binding domains compared with each color representing a different subunit of the homo-tetrameric structure of 3-pGDH. The graph on the left, 1YBA, is a comparison of the x-ray crystallographic data from a native *Escherichia coli* form of 3-pGDH.²⁹ The graph of the right is a mutant, 1SC6, form of *Escherichia coli* form of 3-pGDH. This 1SC6 mutant has a mutation of the 109-tryptophan, which is a residue that lies on the "X" interface between the substrate binding domain and nucleotide binding domain. With this mutant kinetic studies have proven the protein to be relatively dead and inactive but still able to bind the cofactor. Yet, further studies must be conducted to characterize the conformation changes of the Tryptophan mutation.³⁰



It is apparent from these graphs that there is a loss in the subunit cooperativity in the mutant form. The pattern of the A&C subunits of 3-pGDH showing less motility and

²⁹ Protein Data Bank. The active form of phosphoglycerate dehydrogenase. PubMed Thompson, JR. Bell, JK. Bratt, J. Grant, GA. Vmax regulation through domain and subunit changes. The active form of phosphoglycerate dehydrogenase. <http://www.rcsb.org/pdb/explore.do?structureId=1yba>

³⁰ Protein Data Bank. Crystal Structure of W139G D-3-Phosphoglycerate dehydrogenase complexed with NAD⁺ Bell, JK. Grant, GA. Banaszak, LJ. "Multiconformational states in phosphoglycerate dehydrogenase" <<http://www.rcsb.org/pdb/explore.do?structureId=1sc6>>.

therefore tighter binding to the NADH cofactor and the B&D subunits showing a higher B-factor and more motility and less tight binding to NADH is lost in the 1SC6 mutant.

These studies were strengthened with bioinformatics and further x-ray crystal structure analysis. These two forms of 3pGDH, 1YBA and 1SC6 were visualized in pyMol and the relative distances between



specific amino acids in the central “X” interface loop region of the protein were measured. This can be seen in the image to the right in which the pink lines represent the measured distances of the specific residues in the loop region. The residues of interest were the 171 and 190 residues.

Distances in 3pGDH (Taken from 1YBA)

Residues	A Chain	B Chain	C Chain	D Chain
171→190	4.78A	4.22A	4.83A	4.54A
Inter-loop Distances		B & D Chains	A & C Chains	
190→190		4.43A	5.38A	

Distances in 3pGDH (Taken from mutant 1SC6)

Residues	A Chain	B Chain	C Chain	D Chain
171→190	7.60A	7.70A	7.70A	7.60A
Inter-loop Distances		B & D Chains	A & C Chains	
190→190		20.3A	20.2A	

From these two charts it is apparent again that in the 1SC6 mutant that the subunit cooperative binding of the cofactor NADH is possibly linked to this additional loss of different distances between the residues of the loop region. All four subunits of 1SC6 seem to have similar distances around 7.6 A but for the 1YBA native form there is a relative difference between these subunits with the A&C chains having a larger distance between their residues around 4.8 A and the B&D subunits having below 4.5 A. This measured difference is significant because it shows a link between the tightness of the binding of the NADH cofactor to the nucleotide binding domain and increased flexibility

and larger distances between the specific amino acids in the loop “X” interface. This is the core finding that is central and critical to the hypothesis proposed in this scientific research. This data shows that there is a direct connection between the tightness of binding of the co-factor NADH as the nucleotide-binding domain that is possibly influenced or directly influenced by the flexibility within the loops that is thought to be an entropic sink. This entropic sink serves as a possible entropic explanation for the change in free energy of the binding of the NADH cofactor to the two sets of subunits in the 3-pGDH homo-tetrameric structure. This data needs to be further compounded with repeated studies, triplicate measurements and a movement into molecular dynamic studies that evade the complications of working with mutants in the wet lab. There is a long way for this project to go, but the preliminary findings that it presents could be monumental in the current proposed models of proteins dynamics and the possible applications of it.

Research on 3-pGDH is relevant to a greater body of knowledge outside understanding the characteristics and nature of this specific protein. Studying the model system of 3-pGDH is relevant in the greater picture of understanding serine regulated-feedback inhibition. The understanding of this allosteric mechanism will better inform the field of allosteric drug design. The potential for allosteric drug design lies in understanding the contribution of dynamics to allosteric regulation- pGDH is a great model to explore this. Understanding this system could also lead to advancements in animal feed enhancements that seeks to regulate 3-pGDH’s activity to amplify production of tryptophan downstream from serine.

As mentioned previously 3-pGDH converts an important substrate in glycolysis, 3-phosphoglycerate to phosphohydroxy-pyruvate through the reduction of NAD⁺. This reaction is a very important branching point from glycolysis that leads to nucleotide biosynthesis and connects to the TCA cycle and fatty acid biosynthesis. Since 3-pGDH's enzymatic activity creates a specific branch point from glycolysis it is thought that the enzymatic activity of 3-pGDH can possibly be targeted through allosteric drug design. Cancer cells rely strongly on aerobic glycolysis to maintain cell growth and proliferation. The goal of many cancer research groups is to target glycolysis and seek to inhibit it. 3-pGDH offers a strong possible avenue for this research because it is the first step in glycolysis and is inhibited by serine.

Cancer cells rely on aerobic glycolysis to maintain cell growth and proliferation via the Warburg effect. The Warburg effect explains how cancer cells predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells. Malignant tumor cells have glycolytic rates up to 200 times higher than those of their normal tissues of origin. Warburg believed that this change in metabolism was the fundamental cause of cancer. The Warburg effect is thought to be the result of mutations in oncogenes and tumor suppressor genes, which are responsible for malignant transformation.³²

Certain breast cancers are dependent on the overexpression of 3-pGDH. 3-pGDH is expressed in many ER-negative human breast cancer cell lines. In many knockout

³² Hitosugi T¹, Zhou L, Elf S, Fan J, Kang HB, Seo JH, Shan C, Dai Q, Zhang L, Xie J, Gu TL, Jin P, Alečković M, LeRoy G, Kang Y, Sudderth JA, DeBerardinis RJ, Luan CH, Chen GZ, Muller S, Shin DM, Owonikoko TK, Lonial S, Arellano ML, Khoury HJ, Khuri FR, Lee BH, Ye K, Boggon TJ, Kang S, He C, Chen J. "Phosphoglycerate mutase 1 coordinates glycolysis and biosynthesis to promote tumor growth." *Cancer Cell*. Nov 13 2012 22 (5) 585-600.

studies of 3-pGDH it has been shown that there is a correlated reduction in serine synthesis and impairment of cancer cell proliferation.³³

Targeting 3-pGDH could possibly control the growth and proliferation of cancer cells. If this project can identify the mechanism by which serine inhibits the catalytic activity of 3-pGDH, then more information can be provided to develop methods for drug-targeting, and other drug therapies. The idea of allosteric drug design is using allosteric sites of proteins to make flexible regions of proteins less flexible and alter the functioning of a protein.³⁴

³³ Chen J, Chung F, Chen J¹, Chung F, Yang G, Pu M, Gao H, Jiang W, Yin H, Capka V, Kasibhatla S, Laffitte B, Jaeger S, Pagliarini R, Chen Y, Zhou W. "Phosphoglycerate dehydrogenase is dispensable for breast tumor maintenance and growth" *Oncotarget*. Dec 4 2013 (12) 2502-11.

³⁴ Bell, Jessica. Grant, Gregory. Banaszak, Leonard. "Multi-conformational States in Phosphoglycerate Dehydrogenase" *Journal of Biochemistry*. January 23, 2004. American Chemical Society